

Monoclonal Antibodies and Cancer

edited by

George L. Wright, Jr.

*Department of Microbiology and Immunology
and the Immunology Program
Eastern Virginia Medical School
Norfolk, Virginia*

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7 MONOCLONAL ANTIBODIES TO OSTEOGENIC SARCOMA ANTIGENS

Michael Jim Embleton / Cancer Research Campaign Laboratories, University
of Nottingham, Nottingham, England

INTRODUCTION	181
CHARACTERIZATION OF MONOCLONAL ANTIBODIES TO OSTEOGENIC SARCOMAS	184
Characterization and Specificity	
Direct Binding to Target Cells	
Binding Inhibition Assays	
TUMOR-LOCALIZING PROPERTIES	191
Uptake by Osteogenic Sarcoma Xenografts	
External Imaging of Xenografts	
TARGETING OF THERAPEUTIC AGENTS	197
Therapeutic Drugs and Toxins	
Immunomodulators	
CONCLUSIONS AND FUTURE DEVELOPMENTS	203
REFERENCES	205

INTRODUCTION

The detection of antigens associated with human tumors has in the past been imprecise and often fraught with problems of an artifactual nature. This is largely because, apart from studies in which cancer patients have been tested for delayed cutaneous hypersensitivity to tumor extracts, the majority of investigations have employed in vitro methods to assess the reactivity of sera or lymphocytes against tumor cells or subcellular fractions. Assays for cell-mediated cytotoxicity are no longer considered to be appropriate for identifying tumor-associated antigens, because the most efficient mediators of cytotoxicity in most assays are natural killer cells, which are present in both healthy donors and cancer patients to approxi-

Table 1 Detection of Serum Antibodies Against Cell Lines from Two Osteogenic Sarcoma Patients by Indirect Radiolabeled Protein A Assay

Serum donors	Target cell donor M. U.		Target cell donor P. R.	
	Tumor (791T)	Fibroblasts	Tumor (788T)	Fibroblasts
Tumor donor	7/16	6/10	0/14	0/14
Other osteogenic sarcoma patients	4/8	4/8	—	—
Other cancer patients	2/16	—	—	—
Normal control donors	6/41	6/26	0/11	1/19
Multiparous pregnant donors	0/21	0/21	0/15	0/15

mately equal degrees. Other assays for cell-mediated immunity exist, but they have not achieved the popularity at one time attained by cytotoxicity tests.

Serology of human tumors is also beset with problems, but despite this there is evidence indicating that patients with some tumors, including osteogenic sarcoma, are able to mount an immune reaction against their tumor.

There have been a few reports of cell-mediated immunity to osteogenic sarcomas, detected either by cytotoxicity (1,2) or by lymphocyte blastogenesis assays (3,4), but most of the evidence has been obtained by serological methods. The earliest report was in 1968 from Morton and Malmgren (5), who demonstrated antibody reactions by observing cytoplasmic immunofluorescence of fixed cells obtained from imprints of sarcoma tissues. Four osteogenic sarcoma patients were reactive against their own tumor and reactivity was also seen in sera from members of their families (85%) or close associates (91%). Normal donor sera were positive in 29% of the cases. Later studies, mainly employing cultured osteogenic sarcoma cells, have amply confirmed and extended these observations by means of immunofluorescence, complement fixation, and complement-dependent cytotoxicity assays (6-14). On the basis of such work, one group tentatively identified three classes of antigen associated with osteogenic sarcomas, which they designated S1, S2, and S3 (15-18). These antigens were not, however, specific for osteogenic sarcoma.

An overall appraisal of these and later investigations (19-22) reveals several important problems. In many studies it was difficult or impossible to detect antibody activity against fresh osteogenic sarcoma cells, the cells becoming positive

only after a period that several fibroblast sarcoma patients positive with however cells.

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only after culture. Moreover, the expression of antigens tended to fluctuate over a period of time. Even more crucial, in some studies it has been clearly shown that sera from osteogenic sarcoma patients react equally well against cultured fibroblasts and that sera from normal subjects are as reactive as those from sarcoma patients (15,19,20). Data obtained by the author also show this trend (Table 1). Sera from the autochthonous host or allogeneic osteogenic sarcoma patients were reactive against both tumor cells and fibroblasts derived from one patient (M. U.) in a radiolabeled protein A binding assay. Control sera were also positive with both tumor cells and fibroblasts, although the proportion of positives was less than that seen with sarcoma patients. With a second patient (P. R.), however, neither autochthonous nor control sera reacted with cultured target cells.

At one time, cross-reactivity observed between patient and allogeneic tumors and the frequent finding of reactivity by regular associates of the patients were considered to constitute evidence for a viral etiology. However, cross-reactivity with normal sera and normal cells would dismiss this possibility. Various factors have been identified as causing nonspecific reactivity of sera with osteogenic sarcomas. Bloom (13) has shown that mycoplasma infection of target cells increases their tendency to react with human sera, and that cross-reactivity may often be due to the expression of blood group antigens by cultured tumor cells (14). Rosenberg and colleagues claim that cross-reactions such as those observed between osteogenic sarcomas, fibroblasts, and normal sera are due to the expression of fetal antigens in cultured cells (19,20). They were able to show that absorption of normal sera with fetal tissue removed cytolytic activity for both osteogenic sarcoma cells and fibroblasts, and that similar absorption of sarcoma patients' sera removed activity against fibroblasts while antibody lysing tumor cells was retained (21). The suggestion was that osteogenic sarcoma patients had antibodies to tumor-associated antigens in addition to antibodies to fetal antigens, detection of the former being prevented by the cross-reactivity of the latter. However, as shown in Table 1, multiparous donors did not react with osteogenic sarcoma lines 791T and 788T and, furthermore, attempts to absorb antibody with fetal tissue failed (A. S. Eiras and M. J. Embleton, unpublished data). Another suggested cause of cross-reactivity is the presence of antibodies to culture medium components, especially fetal calf serum, which may become incorporated into cell membranes. Thus Irie and Morton (22) identified fetal calf serum as the origin of a new membrane antigen acquired by cultured cells and suggested that human sera often contain antibodies to this component. Yet a further possibility is that immune complexes in sera might bind nonspecifically to cultured cells, and several authors have shown that osteogenic sarcoma patients tend to have high levels of circulating immune complexes (23-26).

In view of the aforementioned problems, the use of the patients' sera for detection of antigens associated with osteogenic sarcoma cannot be considered a practical proposition. Xenogeneic antisera are also unsuitable because it is impossible to be

sure that all nonrelevant antibodies are removed by absorption. In this light, monoclonal antibodies produced by somatic cell hybrids offer an attractive alternative, and it is for this reason that monoclonal antibodies were prepared against osteogenic sarcoma cell line 791T (27,28).

CHARACTERIZATION OF MONOCLONAL ANTIBODIES TO OSTEOGENIC SARCOMAS

Characterization and Specificity

BALB/c mice were immunized against the osteogenic sarcoma cell line 791T by two intraperitoneal injections of 10^7 cells 1 week apart. One week later the mice were given 2×10^6 cells by intracardiac injection and after a further period of 5 days one mouse was sacrificed and its spleen cells were fused with cells of the BALB/c myeloma P3-NS1-Ag-4 (abbreviated to P3NS1) by the method of Galfré et al. (29). The supernatants of the resulting hybridoma cultures were screened for antibody binding to 791T cells using an indirect radioiodinated protein A assay (27). Two supernatants showed preferential binding to 791T cells, but were negative with a small panel of normal fibroblasts or unrelated tumor lines. These two 791T-positive hybridomas were cloned in soft agar and monoclonal antibodies produced by them were tested, using the radioiodinated protein A assay, on a range of target cells, including cultured osteogenic sarcoma cells, other cultured tumor cells, cultured fibroblasts, and some freshly prepared normal cells (Table 2). The most strongly reactive of these antibodies was designated $\alpha 791T/36$, and the other (with a low titer and binding fewer counts per minute of [^{125}I] protein per 10^5 791T cells) was designated $\alpha 791T/48$ (27,28). Antibody $\alpha 791T/36$ bound to 7 of 13 osteogenic sarcoma lines, the strongest reactions occurring with 791T and 788T. Compared with this, $\alpha 791T/48$ bound to only 3 of the 13 lines. Neither antibody reacted with fibroblasts derived from the tumor donor (791SK from skin, or 860 and 870 from cultures of tumor-derived cells) or with fibroblasts from donors of cross-reactive osteogenic sarcomas (e.g., 788SK and 805SK). Reactions with lymphocytes and erythrocytes from a range of donors were also negative. Immunoperoxidase staining of frozen sections also revealed no reactivity of $\alpha 791T/36$ with human liver, lymphoid, brain, or breast tissue. It thus appeared that both monoclonal antibodies recognized antigens expressed on tumors rather than on normal cells, although it cannot be discounted that more exhaustive testing might reveal reactivity against some normal components.

Cross-tests on cells cultured from other tumor types indicated that neither antibody was specific for osteogenic sarcomas. Antibody $\alpha 791T/36$ was strongly positive for prostate carcinoma EB33 and HeLa cells, and more weakly reactive with colon carcinoma lines HT29, Loach, and LS174T, and lung carcinoma A549.

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Antibody α 791T/48 cross-reacted with none of these lines, but reacted with breast carcinoma SKBr3 and a fibroblastic fetal bone marrow line, 74BM. The only cell lines against which both hybridomas were reactive were osteogenic sarcomas 791T and 788T. This strongly suggests that the two monoclonal antibodies recognized separate epitopes, which both happen to be shared by 791T and 788T, but are otherwise randomly distributed among other tumor cell lines and possibly some fetal cells.

Absorption studies confirmed the cross-reactivity of α 791T/36 determined in the direct tests. In the absorption tests, aliquots of supernatant diluted 1:10 were incubated with various cell lines, at 10^8 cells per milliliter, before being tested for antibody activity against 791T target cells or the cell line used for absorption. The results (Table 3) indicated that absorption with any of the cross-reactive cell lines could remove antibody reactive with both 791T and the absorbing cells, but absorption with non-cross-reactive cells (Mel-2a, Mel-57, and PA-1) had little effect. The positive cross-reactions were therefore due to a shared epitope rather than nonspecific antibody uptake.

Purification of both antibodies was achieved by affinity chromatography on protein A linked to Sepharose 4B. Ascites lines prepared by passaging the cloned hybridomas in BALB/c mice produced antibody concentrations in ascites fluid 50-100 times that in culture supernatants. The subclasses of the two preparations were determined by radioisotopic assays in which purified antibody was adsorbed to vinyl microtiter plates, followed by reaction with ^{125}I -labeled specific subclass antisera. By this means it was determined that both were IgG_{2b} , and also that both hybridoma supernatants contained the mouse kappa light chain characteristic of the P3NS1 myeloma.

Since IgG_{2b} is a complement-fixing subclass, it was of interest to determine whether the monoclonal antibodies could mediate complement-dependent cytotoxicity. This was established using a ^{51}Cr release assay employing rabbit complement (Fig. 1). Both antibodies were cytotoxic in the presence of complement, this reactivity being greater with α 791T/36. Cytolysis of different target cells followed exactly the same specificity as previously established in [^{125}I] protein A binding assays.

Direct Binding to Target Cells

To directly detect the binding properties of α 791T/36 and α 791T/48, the monoclonal antibodies were labeled with ^{125}I , using the chloramine T procedure (30), giving preparations with a specific activity of about $10 \mu\text{g}/\text{mg}$ protein. The specificity of binding of these labeled products was tested on a selected range of target cells, as shown in Table 4. The sensitivity of direct binding by labeled antibody was much less than that of the indirect protein A binding assay, but on the whole the previously established specificity was preserved. Antibody α 791T/36

Table 2 Cells Reacting with Monoclonal Antibodies Against Osteogenic Sarcoma Line 791T

Tissue type	Reactions with antibody α 791T/36		Reactions with antibody α 791T/48	
	Positive	Negative	Positive	Negative
Osteogenic sarcoma	791T ^a	781T	791T	845T
	788T ^b	803T	788T	805T
	845T	836T	781T	2 OS
	805T ^c	706T		T278
	2 OS	888T		393T
	T278	792T		803T
	393T			836T
				206T
Colon carcinoma	HT29	HCT8		888T
	Loach	HRT18		792T
	LS174T			HT29
				HCT8
Breast carcinoma		734B	SKBr3	HRT18
		SKBr3		Loach
		HS578T		734B
		MCF-7		HS578T
Lung carcinoma	A549	A427		MCF-7
		9812		A549
				A427
Prostate carcinoma	EB33			9812
Cervix carcinoma	HeLa			EB33
Bladder carcinoma		T24		HeLa
Ovarian carcinoma		PA-1		T24
Melanoma		MeWO		PA-1
		Mel-2a		MeWO
		Mel-57		Mel-2a
		NK1-4		Mel-57
		RPMI 5966		NK1-4
		Mel-Swift		RPMI 5966
Lymphoblastoid cells		Raji		Mel-Swift
		K562		Raji
				K562

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Table 2 (Continued)

h '/48 gative	Skin fibroblasts	791SK ^a	791SK
		788SK ^b	788SK
		805SK ^c	803SK
		803SK	181SK
		836SK	
		181SK	
	Lung fibroblasts	618Lu	618Lu
	Tumor-derived	860 ^a	860
	fibroblasts	870 ^a	870
	Fetal bone marrow	181BM	74BM
		74BM	
	Freshly prepared	Erythro-	Erythro-
	normal cells	cytes	cytes
		Leukocytes	Leukocytes
	Frozen sections ^d	Liver	
		Spleen	
		Brain	
		Breast	

^aThese lines were derived from donor M. U.^bThese lines were derived from donor P. R.^cThese lines were derived from donor Q. L.^dFrozen sections were tested by immunoperoxidase staining.

bound strongly to 791T, 788T, and HeLa cells, although weakly reactive cell lines (A549, HT29) gave only background levels of binding, no higher than seen with cell lines known to be non-cross-reactive with 791T (Table 2). Binding of labeled α 791T/48 was very weak compared with α 791T/36, but nevertheless it bound more strongly to 791T and 788T than to other cell lines. It was, however, negative with breast carcinoma SKBr3, to which it was reactive in indirect protein A tests.

Using directly labeled antibodies, and knowing that the specificity of binding to the strongly reacting cell lines was unchanged by the radioiodination procedure, it was possible to make an estimate of the number of antibody binding sites on 791T target cells. Cells were incubated with increasing quantities of radiolabeled antibody until saturation was achieved. Saturation corresponded to binding of 2.2×10^6 IgG molecules per cell in the case of α 791T/36, and 2×10^5 IgG molecules per cell in the case of α 791T/48, using calculations devised by Fazekas de St. Groth (31). Osteogenic sarcoma 788T and HeLa cells bound 1.6×10^6 and 1.9×10^6 α 791T/36 IgG molecules per cell, respectively, and more weakly

Table 3 Absorption of $\alpha 791T/36$ Monoclonal Antibody by Cultured Tumor Cells

Absorbing cells	Percentage of absorption ^a of antibody reacting with target cells				
	791T	788T	EB33	HeLa	A549 HT29
791T osteogenic sarcoma	97	91	94	90	96 76
788T osteogenic sarcoma	92	93			
EB33 prostate carcinoma	95		94		
HeLa cervix carcinoma	94			92	
A549 lung carcinoma	100				75
HT29 colon carcinoma	70				70
Mel-2a melanoma ^b	24				
Mel-57 melanoma	15				
PA-1 ovarian carcinoma	11				

^aAbsorption was accomplished by incubating 10^8 cells per milliliter of hybridoma supernatant (diluted 1:10) for 1 hr at 37°C . Cells were removed by centrifugation and the absorbed supernatant was tested using a [^{125}I] protein A binding assay (27).

^bMelanomas Mel-2a and Mel-57 and ovarian tumor PA-1 were not cross-reactive with $\alpha 791T/36$ (Table 2).

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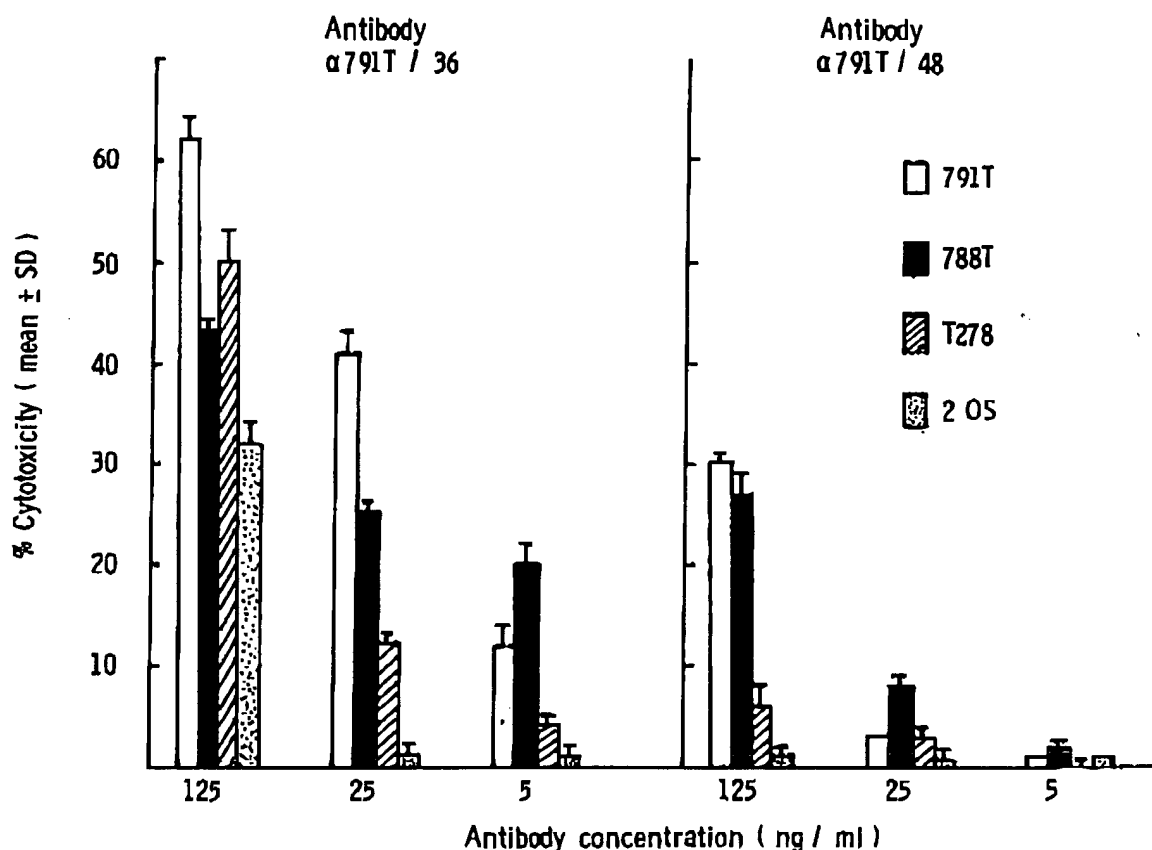


Figure 1 Complement-dependent cytotoxicity against human tumor cells by two monoclonal antibodies to osteogenic sarcoma line 791T. Aliquots of 10^4 ^{51}Cr -labeled cells were incubated with affinity-purified monoclonal antibody (100 μl per well) in round-bottom microtiter plates and 100 μl of rabbit serum were added as a source of complement. After 2 hr. of incubation at 37°C the percentage release of ^{51}Cr was estimated by measuring the radioactivity of supernatant samples. Culture medium was used as a negative control and cells exposed to 1% sodium dodecyl sulfate provided a "maximum release" figure. The percent of cytotoxicity was calculated as

$$\frac{\%^{51}\text{Cr release in test} - \%^{51}\text{Cr release in controls}}{\text{maximum } \%^{51}\text{Cr release} - \%^{51}\text{Cr release in controls}} \times 100$$

Complement alone or antibody with heat-activated complement were not cytotoxic.

reactive osteogenic sarcomas (T278, 393T, and 2 OS) bound between 4.3×10^5 and 5.3×10^5 . These cells did not produce detectable cross-reactions with $\alpha 791\text{T}/48$ in previous tests (Table 2) and the number of $\alpha 791\text{T}/48$ IgG molecules they bound was between 2×10^4 and 4×10^4 molecules of $\alpha 791\text{T}/48$ per cell. These low levels of binding probably represent nonspecific protein adsorption.

Table 4 Binding of Radioiodinated Monoclonal Antibodies to Tumor Target Cells

Target cell	Tumor type	Mean percentage of labeled antibody bound	
		791T/36	791T/48
791T	Osteogenic sarcoma	7.85	0.78
788T	Osteogenic sarcoma	8.83	0.52
888T	Osteogenic sarcoma	0.89	NT ^a
HeLa	Cervix carcinoma	8.07	0.27
HCT8	Colon carcinoma	0.91	0.22
HRT18	Colon carcinoma	1.12	0.14
HT29	Colon carcinoma	0.75	0.24
A549	Lung carcinoma	1.11	0.32
A427	Lung carcinoma	0.70	0.25
T24	Bladder carcinoma	0.97	0.18
EB33	Prostate carcinoma	NT	0.36
PA-1	Ovarian carcinoma	0.32	NT
SKBr3	Breast carcinoma	0.47	0.38
RPMI 5966	Melanoma	1.50	0.22

^aNT, not tested.**Binding Inhibition Assays**

The differing target cell specificities of the two anti-791T monoclonal antibodies indicate that they probably react with different epitopes. Confirmation of this was obtained in experiments in which the binding of the radioiodinated antibody (50 μ g protein per 10^5 cells) was inhibited by pretreating the target cells with unlabeled antibody (4 mg per 10^5 cells). In these experiments the effects of "cold" α 791T/36 and α 791T/48 were compared with the effects of culture medium (or P3NS1 culture supernatant) or unrelated monoclonal antibodies (Table 5). Binding of 125 I-labeled α 791T/36 to 791T, 788T, and HeLa target cells was inhibited by "cold" α 791T/36, but not by α 791T/48 or two monoclonal antibodies raised against a melanoma and a colorectal carcinoma cell line. Conversely, binding of labeled α 791T/48 was inhibited only by nonlabeled α 791T/48 and was not affected by prior incubation of 791T or 788T target cells with α 791T/36 or unrelated antibodies. It is thus clear that the two antibodies react with

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Table 5 Inhibition of Binding of Radioiodinated Monoclonal Antibodies to Cells Pretreated with Unlabeled Antibodies

Target cell	Blocking antibody	Mean percentage of inhibition of binding of	
		α 791T/36	α 791T/48
791T	SC3982 ^a	< 7.8	NT ^b
	α HTR18/2/33b ^c	< 1.3	5.3
	α 791T/36	80.0	< 5.3
	α 791T/48	< 9.3	62.6
788T	SC3982	9.2	NT
	α HRT18/2/33b	6.1	5.0
	α 791T/36	71.2	< 1.5
	α 791T/48	< 9.5	67.0
HeLa	SC3982	10.5	
	α HRT18/2/33b	K 6.0	
	α 791T/36	68.3	
	α 791T/48	5.9	

^aAnti-melanoma monoclonal antibody.
^bAnti-colon carcinoma monoclonal antibody.
^cAnti-colon carcinoma.

separate epitopes, although these studies do not discern whether these epitopes are present on separate molecules or represent different portions of the same molecule.

Blocking tests were extended to serum from the donor of osteogenic sarcoma 791T (M. U.) in order to determine whether the epitope recognized by α 791T/36 was also recognized by the patient (Table 6). In these experiments, autochthonous and control sera with known positive or negative activity against 791T cells in [¹²⁵I] protein A binding tests were preincubated with 791T target cells before incubation of target cells with ¹²⁵I-labeled α 791T/36 monoclonal antibody. None of the sera tested inhibited binding of [¹²⁵I] α 791T/36. It was thus apparent that antibodies that were present in some of the sera were not directed against the component recognized by the monoclonal antibody.

TUMOR-LOCALIZING PROPERTIES

Uptake by Osteogenic Sarcoma Xenografts

Monoclonal antibody 791T/36 binds much more strongly to 791T cells than α 791T/48 and a larger number of molecules are bound at saturation, so this

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Table 6 Inhibition of Binding of Radiolabeled α 791T/36 Monoclonal Antibody by Tumor Donor Serum

Blocking serum		^{125}I binding ratio ^a in indirect protein A assay	Percentage of inhibition of binding of ^{125}I -labeled α 791T/36
Normal donor serum			
	1	1.15	0
	2	0.92	0
	3	1.52	7
	4	8.27 ($P < 0.001$) ^a	3
Tumor host serum			
	1	2.80 ($P < 0.05$)	8
	2	3.30 ($P < 0.001$)	2
	3	1.19	1
	4	1.00	10
	5	2.82 ($P < 0.05$)	9
	6	1.80	2
	7	1.40	-3

^a Binding ratio = mean cpm bound by cells treated with serum \div mean cpm bound by cells treated with culture medium.

^b Statistical significance assessed by Student's *t* test.

antibody was chosen as a reagent to explore *in vivo* localization to 791T tumors growing in immunodeprived mice. Mice of the CBA strain were subjected to thymectomy, treatment with cytosine arabinoside, and whole body gamma irradiation according to the method published by Steel et al. (32). These mice develop progressively growing tumors 2 or 3 weeks after subcutaneous injection of 10^6 791T cells. Cells obtained by dissociation of 791T xenografts were assayed for expression of the α 791T/36 defined antigen by fluorescence-activated cell sorting using α 791T/36 conjugated to fluorescein isothiocyanate (FITC). Gating conditions for neoplastic cells were established using cultured 791T in order to exclude erythrocytes, infiltrating host lymphoid cells, and stromal cells contained among cells from the xenografts. The FITC-labeled α 791T/36 bound to all the tumor cells. Addition of a fourfold excess of unlabeled α 791T/36 during the initial staining procedure reduced the mean fluorescence per cell to about 18% of control level (expected reduction 20%). It was thus clear that 791T cells continue to express the monoclonal antibody-defined antigen when growing as a xenograft.

Radiodination of α 791T/36 antibody with ^{125}I or ^{131}I was accomplished using the iodogen procedure (33) and preparations of specific activities of 0.25-1.0 $\mu\text{Ci}/\text{mg}$ protein were used. Labeled antibody was injected intraperitoneally into the immunodeprived mice with or without 791T tumors, and 24 hr later their serum

^{125}I Tissue-blood ratio (mean \pm SD)

Figure 791T with tumor gram percent activity

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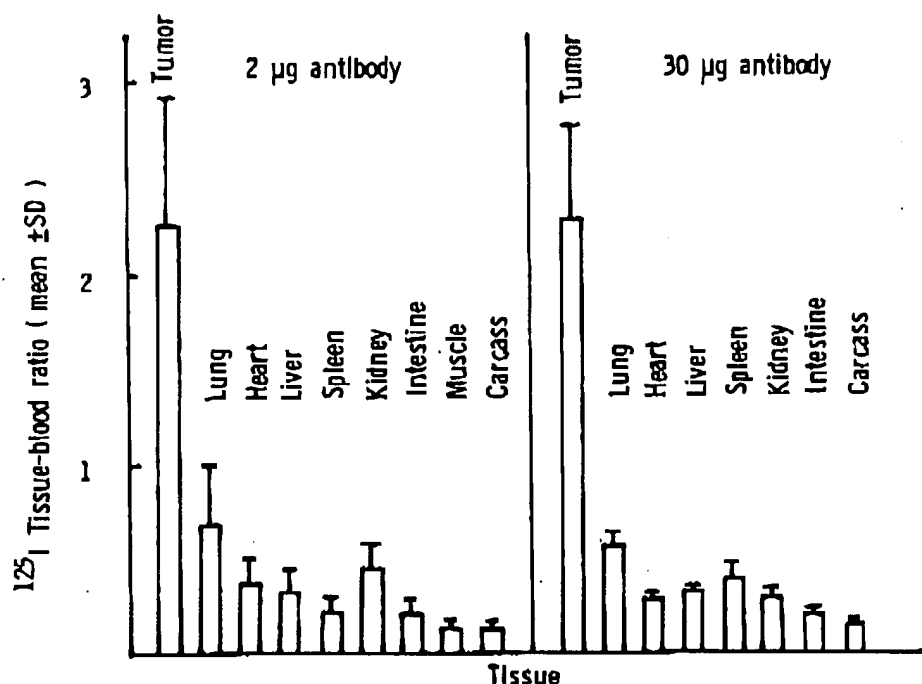
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Figure 2 Localization of monoclonal antibody $\alpha 791T/36$ in osteogenic sarcoma 791T xenografts. Mice bearing 791T xenografts were injected intraperitoneally with ^{125}I -labeled $\alpha 791T/36$ at 2 doses (2 and 30 μg). Three days later blood, tumor, and various organs were removed and the percentage of radioactivity per gram of tissue was measured. The tissue/blood ratio was calculated as the mean percentage of radioactivity per gram of tissue divided by the percentage of radioactivity per gram of blood.

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was removed and chromatographed on Sephacryl 300, in comparison with a mixture of labeled $\alpha 791T/36$ and normal mouse serum. In all three cases the elution profile was similar, radioactivity being associated with a peak corresponding to mouse immunoglobulin. Furthermore, radioactivity could be precipitated with rabbit anti-mouse immunoglobulin from serum of mice inoculated with radioiodinated $\alpha 791T/36$ and antibody in these sera was able to bind directly to 791T target cells. The antibody was therefore not grossly aggregated or degraded following injection into mice.

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For organ distribution studies, the labeled antibody was similarly injected intraperitoneally into 791T tumor-bearing mice and 1-6 days later mice were sacrificed. They were exsanguinated and their tumor, visceral organs, muscle, and bone tissue were removed for estimation of radioactivity in a gamma counter. The activity was expressed as the percentage of injected radioactivity per gram of tissue, divided by the percentage of radioactivity per gram of blood. Tumors were examined 12-30 days after injection of cells (tumor mass 45-201 mg) and in all

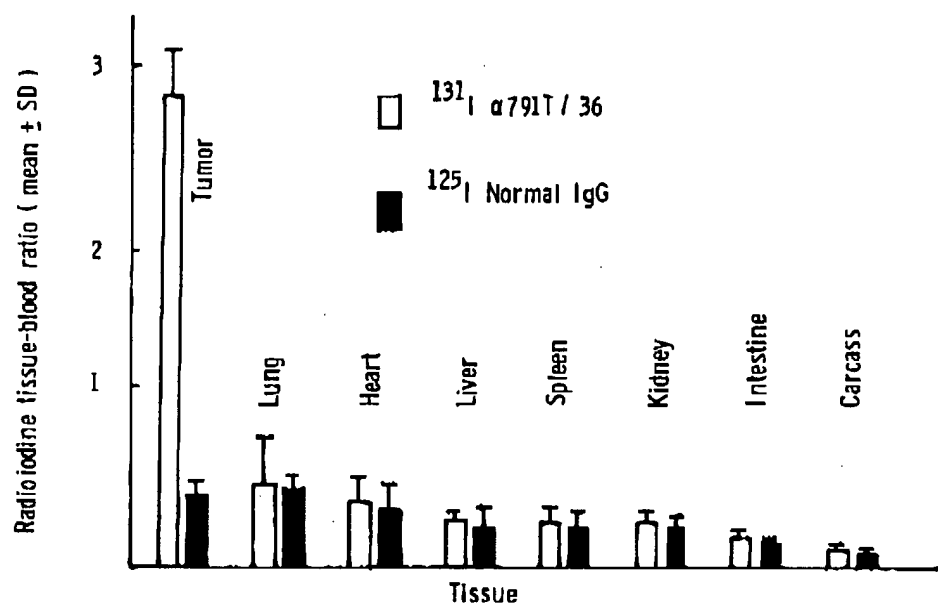


Figure 3 In vivo distribution of radiolabeled normal immunoglobulin compared with monoclonal antibody in 791T xenografted mice. Mice bearing 791T xenografts were injected with ^{131}I -labeled $\alpha 791\text{T}/36$ and ^{125}I -labeled normal mouse IgG simultaneously. Three days later tissue/blood ratios for tumor and various organs were calculated as in Figure 2.

cases the tumor/blood ratios of radioactivity were significantly greater than ratios obtained with any of the normal tissues or the residual carcass. Figure 2 shows the organ distribution at 3 days after administration of ^{125}I -labeled 791T/36 at doses of 2 or 30 μg protein. The organ exhibiting the highest localization apart from the tumor was lung, and at both antibody doses the activity in the lung was much lower than in the tumor on a weight basis. Repeated tests with antibody at doses between 2 and 50 μg per mouse confirmed these findings. In some experiments mice were simultaneously inoculated with ^{131}I -labeled 791T/36 and ^{125}I -labeled normal mouse IgG_{2b}. In this case, the tissue/blood ratios of normal immunoglobulin were low for the tumor and all normal tissues examined (Fig. 3), while the ratios for ^{131}I -labeled $\alpha 791\text{T}/36$ were at a similar low level for normal tissues but high for 791T. Localization within 791T tissue was therefore not due to physical characteristics of the tissue which might result nonspecifically in preferential accumulation of labeled immunoglobulin.

Further specificity checks were carried out with tumors, the reactivities of which were known in $\alpha 791\text{T}/36$ binding tests (Table 2). Osteogenic sarcoma lines 788T and 2 OS, colon carcinoma line HCT8, and bladder carcinoma line T24 were also grown as xenografts, and the mice were inoculated with ^{125}I -labeled $\alpha 791\text{T}/36$. Three days later their tumors and other organs were removed and the tissue/blood ratios of activity calculated. Osteogenic sarcomas 788T and

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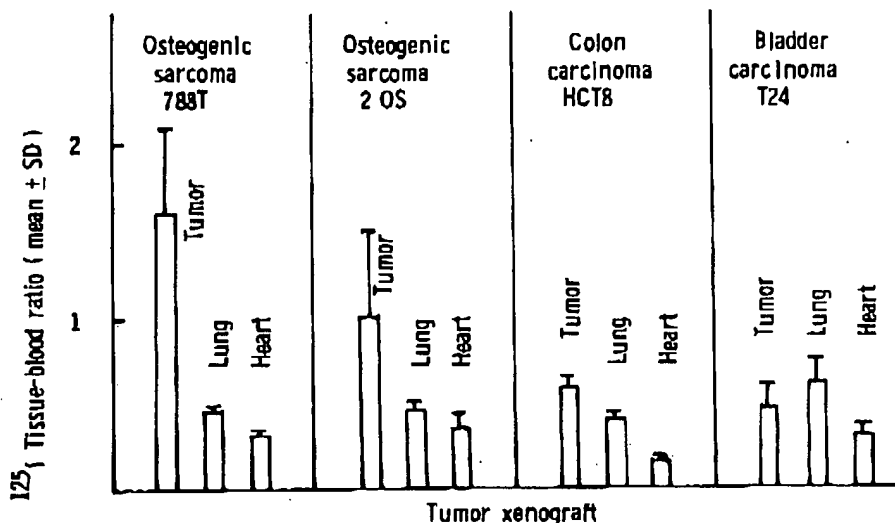


Figure 4 Localization of $\alpha 791\text{T}/36$ monoclonal antibody in tumors other than osteogenic sarcoma 791T. Mice bearing tumor xenografts were injected with ^{125}I -labeled $\alpha 791\text{T}/36$. Three days later tissue/blood ratios were calculated (as in Figure 2) for tumor and various organs, of which only lung and heart are shown. Liver, kidney, spleen, intestine, muscle, and residual carcass ratios were no higher than that shown for heart in each case. Antibody localization occurred in osteogenic sarcomas 788T and 2 OS, but not in colon carcinoma HCT8 or bladder carcinoma T24.

2 OS exhibited higher ratios than normal tissues (Fig. 4), but the non-cross-reactive lines HCT8 and T24 had ratios equivalent to those in normal tissues. In view of the fact that preferential tumor localization occurred only with tumors that react antigenically with the antibody, it was concluded that this was a true antibody phenomenon rather than due to nonspecific uptake by some tumors.

External Imaging of Xenografts

Mice bearing 791T tumor xenografts were given $20\mu\text{Ci}$ of ^{131}I -labeled $\alpha 791\text{T}/36$ intraperitoneally and 48 hr later they were anesthetized with thiopentone. Gamma scintigraphy was carried out using a parallel hole collimator (400 KeV maximum), in which case mice were placed to facilitate an anterior view, or a pinhole collimator, in which case a posterior view was chosen. Distribution of radioactivity within the blood was assessed by intravenous injection of $^{99\text{m}}\text{Tc}$ pertechnetate ($20\mu\text{Ci}$) and images of ^{131}I and $^{99\text{m}}\text{Tc}$ were acquired. Views were stored and processed by computer in a 64×64 or 128×128 cell matrix. Image enhancement by computerized subtraction of $^{99\text{m}}\text{Tc}$ counts from ^{131}I counts clearly delineated subcutaneous 791T growths as areas of increased radioactivity (Fig. 5). Localization using the gamma camera was expressed as the ratio of counts within the region of interest around the tumor, divided by a region of

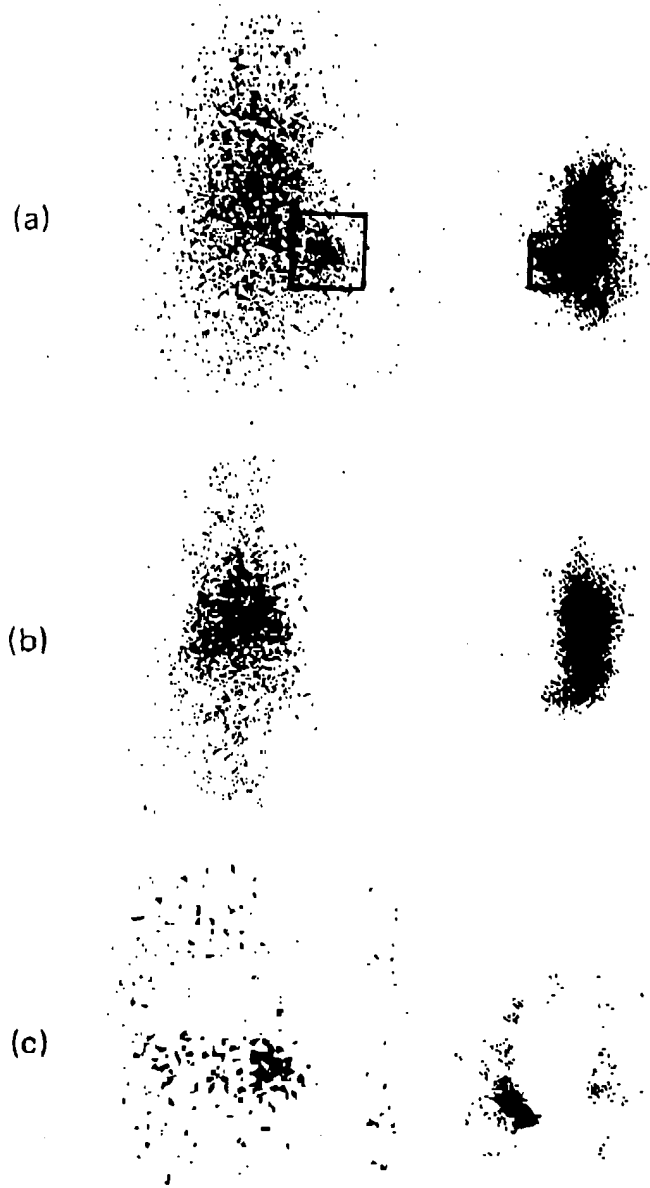


Figure 5 In vivo localization of $\alpha 791T/36$ monoclonal antibody in osteogenic sarcoma 791T xenografts demonstrated by external gamma scintigraphy. Mice bearing 791T xenografts were injected intraperitoneally with ^{131}I -labeled $\alpha 791T/36$ and three days later they were scanned on a gamma camera. Intravenous injection of $[^{99m}Tc]$ pertechnate was also performed in order to acquire an image due to the blood pool. (a) Image acquired with $[^{131}I]\alpha 791T/36$. (b) Image acquired with $[^{99m}Tc]$ pertechnate. (c) Computerized subtraction (a - b) showing enhanced image of tumor. The delineated area is the "region of interest" referred to in the text. The images here were obtained with two separate mice.

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equivalent area on the contralateral side of the mouse. Typically this ratio was around 2:1 using [^{131}I] α 791T/36 only, and after subtraction of $^{99\text{m}}\text{Tc}$ it ranged up to 10:1. At this level of localization, achieved in larger tumors, between 11 and 13% of injected ^{131}I became localized within the tumor.

TARGETING OF THERAPEUTIC AGENTS

Therapeutic Drugs and Toxins

Knowing that tumor-directed monoclonal antibodies are able to localize in tumors, one of their most interesting potential applications is therapy, either directly by infusion of the purified antibody (34-36) or by using the antibody to direct a cytotoxic agent to the site (37-41). Of the two options, the latter seems at the present time to be more attractive. Passive serotherapy appears to be effective mainly with lymphoid tumors, while other tumors may be resistant to monoclonal antibody *in vivo*, although they may be susceptible to antibody-mediated cytotoxicity *in vitro* (42). Osteogenic sarcoma 791T, for example, is not suppressed *in vivo* by treatment of the immunodeprived murine host with α 791T/36 monoclonal antibody.

Several cytotoxic agents have been conjugated to monoclonal antibodies for use as "immunotoxins," these including ricin A chain (37-40), ricin "blocked" by binding to lactose (41), diphtheria toxin A chain (38-40), and adriamycin (43). Using the 791T osteogenic sarcoma as a model, we are currently investigating conjugates prepared with these and a number of other conventional cytotoxic drugs. Most of the conjugates have been prepared using N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) as a coupling agent, although adriamycin has been conjugated to a monoclonal antibody directed against a spontaneous rat tumor by a dextran bridge (43). Experience so far indicates that many agents tend to be inactivated by SPDP, as exemplified in Figure 6, which depicts the relative effects of adriamycin and an SPDP-coupled adriamycin- α 791T/36 conjugate on survival of 791T cells *in vitro*. The cells were cultivated in the presence of the drug or conjugate for 24 hr, followed by labeling of the cells with [^{75}Se] selenomethionine. A radiolabeled amino acid was used with this agent because in preliminary tests high rates of DNA repair led to confusion over the LD_{50} when labeled DNA precursors were used. Uptake of labeled amino acids, however, was correlated extremely well with numbers of surviving cells observed visually. The results (Fig. 6) show that the adriamycin was completely inactivated by direct coupling to the antibody, although in other experiments using dextran as a linking agent it produced therapeutic effects against a rat tumor (43). The ability of the antibody to bind to 791T target cells was hardly affected by the coupling procedure, as indicated by indirect [^{125}I] protein A assays and competition with FITC-labeled α 791T/36 in assays using a fluorescence-activated

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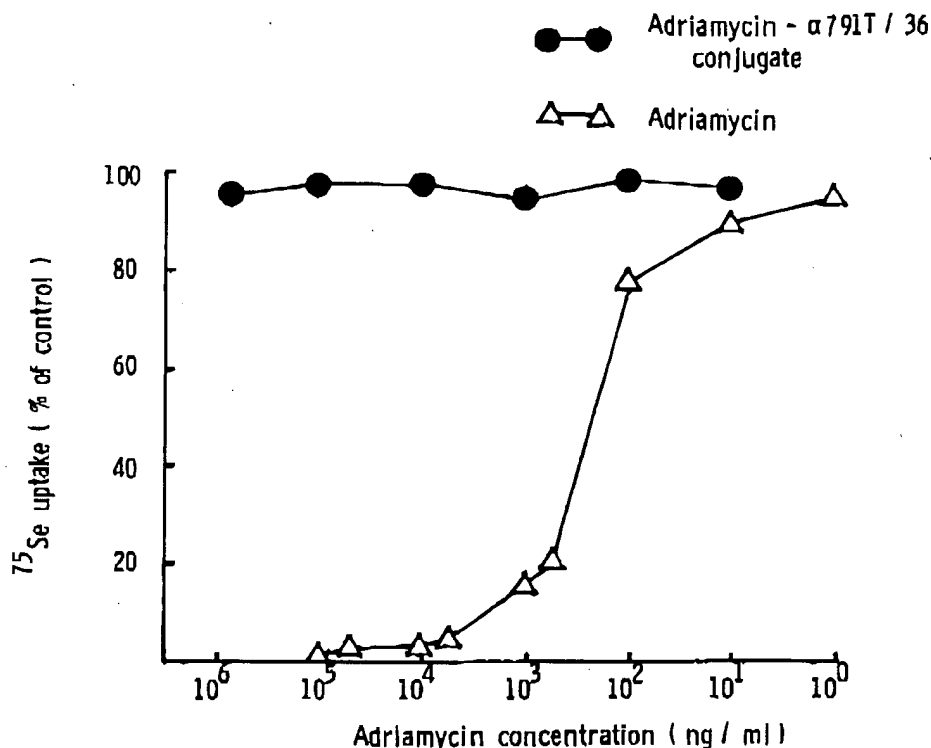


Figure 6 Effect of adriamycin and adriamycin- α 791T/36 conjugate on survival of 791T cells in vitro. Adriamycin or adriamycin- α 791T/36 conjugate (expressed as in nanograms of/adriamycin per millimeter) were added to 791T target cells growing in microtiter plates and the cells were cultured for 24 hr at 37°C. The cells were then labeled with [⁷⁵Se] selenomethionine for 12 hr to assess the relative numbers of surviving cells. Survival is expressed as mean ⁷⁵Se uptake in treated wells calculated as a percentage of that in medium controls. Labeled DNA precursors gave misleading results owing to high rates of DNA repair, but ⁷⁵Se uptake was well correlated with visual scoring of stained preparations.

cell sorter. This has been a common feature of all conjugates, the α 791T/36 antibody retaining between 80 and 100% of its normal activity.

The best SPDP conjugate so far tested with this antibody was prepared with ricin A chain, however, this agent had reduced activity against 791T cells in conjugate form compared with ricin alone when added to growing cultures (Fig. 7), although again antibody titers were only slightly impaired. A similar decrease in toxicity of conjugate compared with parent drug was observed in the case of a conjugate of α 791T/36 and Vindesine (Fig. 8).

When target cells were preincubated with Vindesine-antibody conjugate, however, there was evidence of selective cytotoxic activity against 791T

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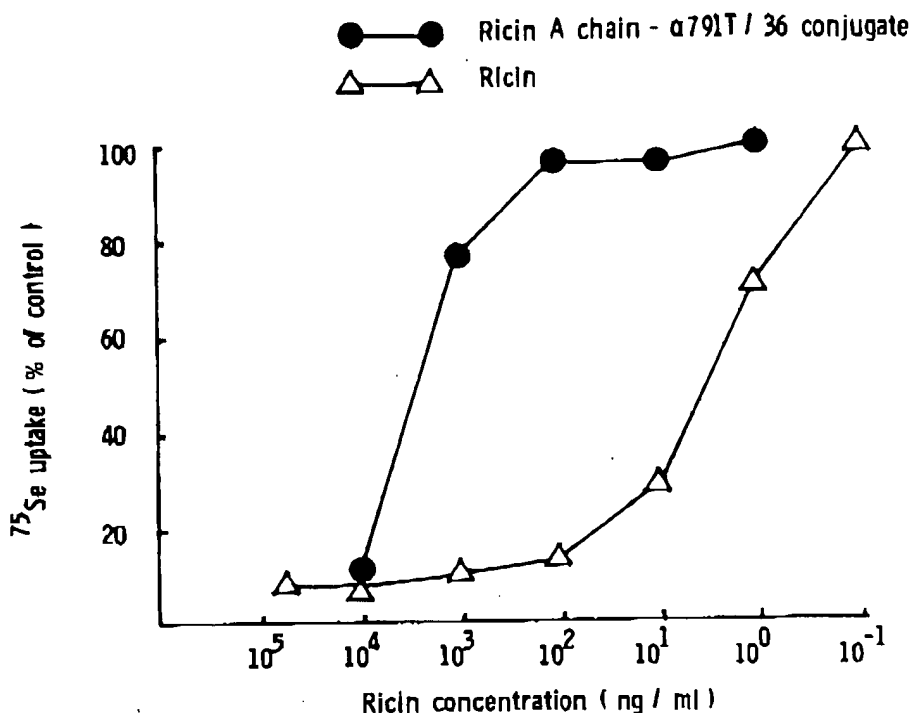


Figure 7 Effect of ricin or ricin A chain-conjugate on survival of 791T cells in vitro. Ricin or ricin- α 791T/36 conjugate were added to 791T cells growing in microtiter plates and the cells were cultured for 24 hr and labeled with [⁷⁵Se] selenomethionine for 12 hr. Survival of 791T cells is expressed as in Figure 6. No ricin A chain was available for comparison.

compared with an antigenically non-cross-reactive melanoma line, Mel-57 (Fig. 9). 791T and Mel-57 target cells were preincubated with the drug or conjugate and washed before plating in microtiter plates. Vindesine alone was toxic to both cell lines, showing no selective activity. The conjugate, on the other hand, was toxic for 791T cells at concentrations that were not significantly active against Mel-57. Similar selective activity on the part of the conjugate was observed with four other cell lines that react with the antibody, but not with four nonreactive lines. This indicates the potential feasibility of selective tumor targeting, although at the present stage of development this is not sufficient to be the basis for an effective therapeutic response. There is obviously a need for the development of alternative coupling methods in order to achieve levels of cytotoxicity with the conjugate equivalent to those obtained with the free drug or toxin, and these studies are in progress.

Immunomodulators

In view of the reported therapeutic effects of interferon (IFN) on osteogenic sarcomas (44) and cytostatic effects observed in vitro (45), it was of interest

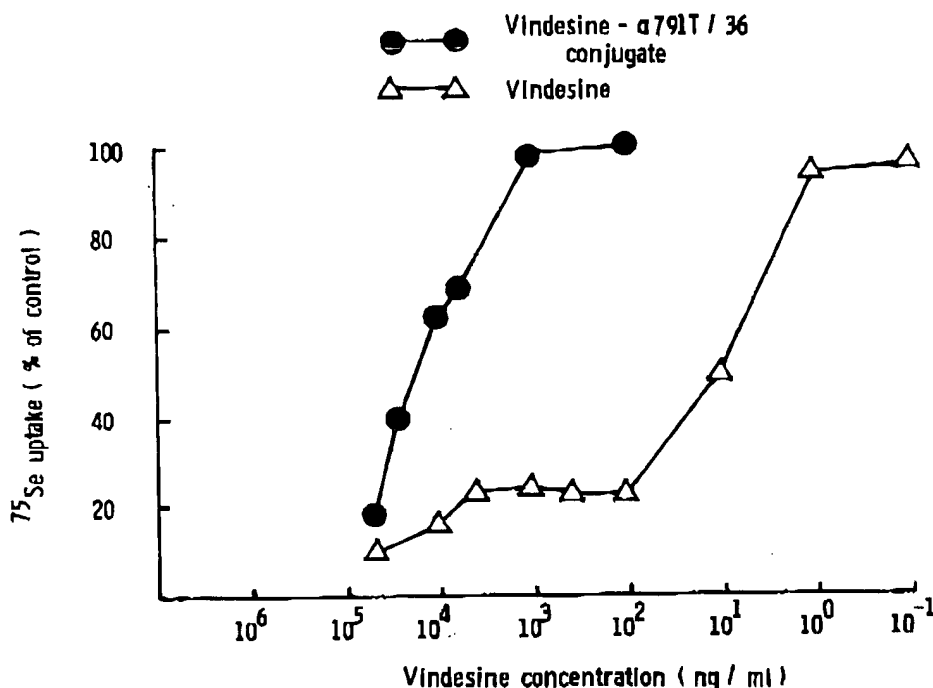


Figure 8 Effect of Vindesine and Vindesine- α 791T/36 conjugate on survival of 791T cells in vitro. Vindesine or Vindesine- α 791T/36 conjugate were added to 791T cells growing in microtiter plates and the cells were cultured for 24 hr and labeled with [^{75}Se] selenomethionine for 12 hr. Survival of 791T cells is expressed as in Figure 6.

to determine the effects of human lymphoblastoid IFN on osteogenic sarcoma 791T. In vitro studies showed no effect of either IFN or IFN- α 791T/36 conjugates on the growth of 791T cells using assays in which cells were cultivated in the presence of IFN for periods of between 1 and 6 days, followed by labeling with [^{125}I] iodeoxyuridine or [^{75}Se] selenomethionine. Interferon also had no effect on the growth of 791T xenografts in Nu/Nu mice.

Since 791T was not directly susceptible to cytostasis by IFN, a decision was made to explore the effect of IFN on cell-mediated cytotoxicity mediated by natural killer (NK) cells. In these experiments peripheral blood lymphocytes from healthy donors were cultured for 6 hr with ^{51}Cr -labeled target cells at effector/target cells ratios 12.5:1, 6.25:1, and 1.56:1. When the percentage of cytotoxicity is plotted against effector/target ratios, slopes of regression fitted through the origin and the plots are directly proportional to the frequency of cytotoxic cells in the effector population (46). The effect of IFN was assessed by incubating the lymphocytes for 1 hr at 37°C with IFN (200 units/ml) followed by three

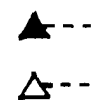
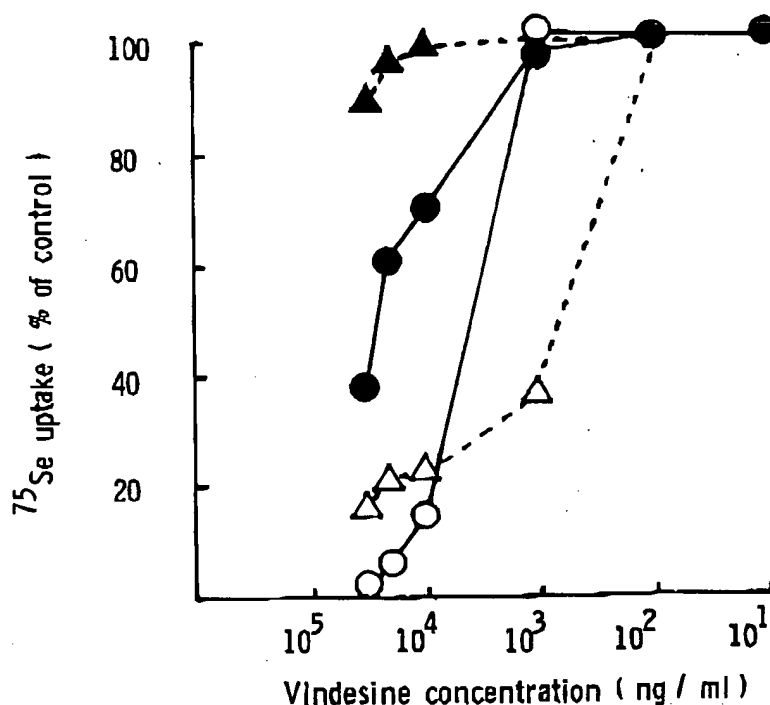


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Figure 9 Effect of preincubation of target cells with Vindesine-α791T/36 conjugate. 791T or Mel-57 cells were incubated with Vindesine or Vindesine-α791T/36 conjugate for 15 min, then washed. Cells were plated at 5×10^3 per well in microtiter plates and cultured for 48 hrs, then labeled for 8 hr with [^{75}Se] selenomethionine. Survival of cells is expressed as in Figure 6.

washes, before addition to the target cells. Direct augmentation of NK cytotoxicity by IFN-α791T/36 conjugate (at 200 units/ml IFN) for two target cell lines is shown in Table 7. Erythroleukemia line K562 is a commonly used target in NK cell assays owing to its high sensitivity to NK cells: peripheral blood lymphocytes alone were cytotoxic for K562, and prior incubation with IFN or

Table 7 Augmentation of Natural Killer Cell Activity in Peripheral Blood Lymphocytes by Interferon-Monoclonal Antibody Conjugate

Target cells	Effector cell treatment	Cytotoxicity (slope \pm S.D.)	Augmentation (%) ^a
K562	Culture medium	8.23 \pm 0.20	—
	IFN	19.31 \pm 0.34	234.8 (P < 0.001)
	IFN- α 791T/36 conjugate	11.55 \pm 0.22	140.4 (P < 0.001)
791T	Culture medium	2.15 \pm 0.29	—
	IFN	8.94 \pm 1.10	415.0 (P < 0.001)
	IFN- α 791T/36 conjugate	6.78 \pm 0.16	315 (P < 0.001)

^aStatistical significance assessed by Student's test

IFN- α 791T/36 conjugate markedly enhanced this cytotoxicity. With ⁵¹Cr-labeled 791T target cells lymphocytes alone had little cytotoxic effect, but this was augmented to a significant level by IFN or the conjugate.

This experiment indicated that IFN carried to the tumor site might have the property of locally activating NK cells against the tumor cells. This view is supported by a more exacting experiment in which 791T target cells preincubated with the IFN- α 791T/36 conjugate were cultured with normal lymphocytes and ⁵¹Cr-labeled K562 cells (Table 8). When the 791T cells had been preincubated with IFN mixed with α 791T/36, they did not augment NK cytotoxicity against labeled K562 cells, but when 791T cells coated with the SPDP-prepared conjugate were tested, they produced augmentation of cytotoxicity equivalent to that observed when lymphocytes were exposed directly to IFN (Table 7). When T24 bladder carcinoma cells, nonreactive with α 791T/36, were pretreated with the conjugate, they induced no augmentation of cytotoxicity for K562 in mixed culture (Table 8). Thus NK cell cytotoxicity against third-party target cells was augmented in these assays by IFN bound to 791T cells by the attached antibody. It could therefore be predicted that the IFN-monoclonal antibody conjugate, localizing in the tumor, might bind to some tumor cells and locally recruit cytotoxic cells against themselves and surrounding tumor cells.

The role of NK cells in tumor rejection is not firmly established and it is likely that cellular effector mechanisms involve a complex interplay of cells, but this does not compromise the possible beneficial effects of a targeted immunomodulator, because these agents may have a stimulating action on diverse effector cells. Because of the species specificity of human IFN, it has not been possible to test the conjugate in vivo against xenografts, where responding cells would be murine in

Table 8 K562 b

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Table 8 Augmentation of Natural Killer Cell Activity Against ^{51}Cr -Labeled K562 by 791T Cells Coated with Interferon-Monoclonal Antibody Conjugate

Tumor cell pretreatment	Treated cells	Cytotoxicity (slope \pm S.D.)	Augmentation (%)
Culture medium	791T	6.68 \pm 0.39	—
IFN and α 791T/36	791T	7.46 \pm 0.57	111.7 (NS) ^a
mixture			
IFN- α 791T/36 conjugate	791T	14.36 \pm 2.04	214.9 (P < 0.01) ^b
Culture medium	T24	8.23 \pm 2.0	—
IFN and α 791T/36	T24	7.81 \pm 0.19	94.9 (NS)
mixture			
IFN- α 791T/36	T24	8.69 \pm 0.33	105.6 (NS)

^a NS, not significant^b Statistical significance assessed by Student's t test.

origin; however, the basic approach would be applicable to virtually any immunomodulatory agent.

CONCLUSIONS AND FUTURE DEVELOPMENTS

The introduction of the hybridoma technique for producing monoclonal antibodies against tumors was heralded by many to provide an answer to whether or not human tumor-associated antigens really exist. This question has been answered to only a limited extent, however, in that the majority of anti-tumor monoclonal antibodies have been produced from immunized xenogeneic, usually murine, spleen cell donors. Some authors have raised human monoclonal antibodies to tumors by creating human-mouse hybridomas (47-49), in which case the fused lymphocytes were derived from cancer patients. Under these conditions antibodies produced by the hybrids can be regarded as products of a host immune response toward the tumor. Immunized mice, however, are capable of recognizing a wider range of antigens on the immunizing cells. Hybridomas producing unwanted antibodies (e.g., against normal adult cells) can be discarded after screening, but there is no guarantee that apparently tumor-associated antibodies are directed toward components that are recognized as an antigen by the host. Indeed, with osteogenic sarcoma 791T there was evidence to the contrary (Table 6). In other words, antigens detected by xenogeneic monoclonal antibodies tell us nothing about the possibility of host resistance to cancer; rather, they sidestep the issue of host immunity and offer an alternative approach toward the application of immunology to human cancer.

Human monoclonal antibodies have a theoretical advantage over xenogeneic monoclonal antibodies in that they might be better tolerated when administered to patients, but in studies where murine monoclonal antibodies have been infused into patients, they have induced no obvious ill effects (35, 36), so this may not be a real advantage. On the other hand, since production of murine monoclonal antibodies does not rely on a pre-existing host immune response to the tumor, it probably stands a better chance of success in the first instance. For biochemical studies the suitability of xenogeneic monoclonal antibodies depends upon the question being asked. Only human antibodies are likely to identify antigens involved in tumor immunity, but the biochemical study of antigens recognized by xenogeneic antibodies should nevertheless provide useful information on tumor cell membrane functions. Such antibodies can also be used to classify neoplasms, as in the case of leukemias (50). This could lead to a more precise judgment of prognosis and better choice of therapeutic modalities. For use in diagnostic tests and targeting, xenogeneic monoclonal antibodies will probably prove to be perfectly acceptable, provided that tumor specificity is sufficient.

The question of tumor specificity is a difficult one. While some reports claim that certain monoclonal antibodies are specific for a particular tumor type (51, 52), there are also reports indicating that hitherto putatively specific monoclonal antibodies have been found to react with other tumors or certain normal cells (53-55). It is likely that the majority of monoclonal antibodies will follow this pattern, as do the antibodies discussed in this chapter (27). However, they still have potential practical value so long as the antigen detected is expressed preferentially on malignant compared with normal tissue. Limitations posed by weak cross-reactivity with some normal cells will depend upon the importance of those cells relative to clinical benefits to be achieved with a monoclonal antibody.

Caution should be used when interpreting results obtained with monoclonal antibodies to cultured cell lines. Cultured tumor cells may not be antigenically typical of the tumor cell population from which they were derived, and it is well established that new artifactual antigens can occur as a result of culture (21, 22, 56). The cultured osteogenic sarcoma line 791T and the antibodies raised against it constitute a model system, and efforts to produce new anti-tumor monoclonal antibodies in the author's laboratory are now conducted with/fresh, surgically removed tumor specimens. Indeed, in a recent report exactly this approach has been taken with tissue from a biopsy of osteogenic sarcoma (57). Five murine monoclonal antibodies raised against this tissue were tested by immunofluorescence on cryostat sections of various tumor and normal cells, and three of the antibodies appeared to be specific for osteosarcoma and chondrosarcoma, although it is to be noted that they bound weakly to some chondrocytes in apparently normal tissues.

Despite reservations about the use of cultured cells as immunogens, however, model systems lay the foundations upon which future clinical applications are

based. For example, antigen antibody and tumor site antibodies in model system procedures.

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based. For example, radiolabeled xenogeneic (polyclonal) anti-carcinoembryonic antigen antibodies are already being used clinically to determine tumor burden and tumor site by gamma scintigraphy (58), and the hope is that monoclonal antibodies may lead to significant improvements in this field (59). Experimental model systems are necessary to develop optimal ways in which to carry out such procedures.

It is too soon to predict the therapeutic success of anti-tumor agents targeted to tumors by linking to monoclonal antibodies, but the possible methods of coupling and the number of agents available are extensive enough to allow adequate scope for development. As pointed out above, this can be considered something of a side step for tumor immunology in comparison with latter-day attempts to boost the host's own response, but hopefully it may lead to more success.

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